Effect of Organic Acids Produced by *Penicillium notatum* on the Extraction of Metals Ions from Brown Shale

HAQ NAWAZ BHATTI*, SADIA SARWAR AND SADIA ILYAS
Department Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan.
hnbhatti2005@yahoo.com*

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Summary: Preset study was undertaken to check the bioleaching feasibility of brown shale with *Penicillium notatum* by using different carbon sources. Large quantities of the metal ions are embodied in shales that can be recovered. Presently available techniques (pyrometallurgical and hydrometallurgical) are expensive or may have a negative impact on the environment. *Penicillium notatum* exhibited a good potential in generating varieties of organic acids effective for metal ions solubilization. Maximum leaching of Cu (91.55 %) and Mg (79.83 %) was obtained with glucose as substrate while maximum recovery of Mn (71.46 %) was obtained in medium having molasses as substrate.

Introduction

The regular depletion of worldwide reserves for high grade ores is resulting in development of different technologies to recover metal ions from low grade ores and minerals. Though some probable solutions are proposed yet these are not practically suitable as the processes consume huge amount of energy. Another genuine area of concern is the rising cost of environmental protection through out the world. Bioprocessing of minerals for metals is regarded as one of the most convincing solution to the above problems. The technique holds the promise of reducing the fixed capital costs dramatically and also offers the chance to reduce environmental pollution. The biological processes are carried out under ambient conditions and the products of these processes usually end up in aqueous solutions which are more agreeable to control and treatment. The solubilization of metal ions by microorganisms from the mineral ores and the recovery of metal ions from solutions is referred as bioleaching [1].

Bioleaching is a simple, environmental friendly and effective technology for extraction of metal ions from low-grade ores and mineral concentrates using components found readily within the environment [2]. The mining industry, in recent times, has started using a new technique to extract minerals such as gold and copper from mines using an environmental friendly technology like bioleaching technology. Traditionally, the extraction would involve many costly stages such as roasting and smelting process in pyrometallurgical processes [3].

Fungi are predictable to be proficient bioleachers due to their acid secreting potentials. The organism utilizes carbon source and excretes organic acids (oxalic acid, citric acid, gluconic acid etc.) and compounds with at least two hydrophilic reactive groups (e.g. phenolic derivatives) into the culture medium as metabolic products which then reacts with the ore surface, and consequently dissolving heavy metals by forming salts and chelates [4-6]. Heterotrophs require carbon as energy source to produce organic acid metabolites which include citric, oxalic and gluconic acids during bioleaching [7] and this requirement can be fulfilled by using organic wastes. Acidolysis is the principal mechanism in bioleaching with fungal strains like *Penicillium notatum* and *Aspergillus niger*. Agricultural and industrial wastes are among the causes of environmental pollution. Their conversion into useful products may ameliorate the problems they cause. These wastes which include cereals, straw, leaves, seed cake, corncobs, mango peel, rice husk are available abundantly in Pakistan. In most parts of the country, these materials are mainly used as animal feeds. A large quantity is left on farmlands to be decomposed by microorganisms such as bacteria and fungi and produced number of organic acid metabolites like citric, oxalic, malic, gluconic acids used for bioleaching of metals [8].

Shale is a fine-grained sedimentary rock whose original constituents were clay minerals or muds. It is characterized by thin laminae breaking with an irregular curving fracture, often splintery and usually parallel to the often indistinguishable bedding plane. Pakistan has huge reserves of black and brown shale deposits i.e., the Foothills of Himalayas at Lagarbon, in Karakoram valley at Shishi Gol and South of Mirkhani, Kakul in Hazara District, the

Chitral areas as well as the Turbela areas.

The main purpose of present research work was to check the efficiency of organic acids produced by fungus in metal extraction from brown shale by *Penicillium notaum*. Process efficiency was enhanced further by employing low-cost carbon sources as substrates.

Results and Discussions

Physically the shale sample appears to be brown in color, alkaline in nature and insoluble in water. The coloring material in individual shales might be due to carbon, hydrocarbon or finely divided iron sulfide. Mineralogical analysis of brown shale sample contains Monticellite, Mayenite, Calcite, Magnesian, Quartz, Phlogopite and Montmorillonite minerals. While elemental analysis of ore shows the presence of 3.65, 95.8, 146.90, 247.45, 365.35 and 869.00 mg/L of Ni, Mn, Cu, Mg, Al and Ca.

Fig. 1-4 (a-c) depict the results of chemical leaching. In case of copper solubilization, citric acid showed more potential than oxalic acid although oxalic acid is stronger then citric acid [9]. In case of citric acid (0.5 %) about 89.20 mg/L Cu was solubilized while at the same concentration oxalic acid showed 73.60 mg/L solubilization. Tartaric acid solubilized 69.09 % Cu with 0.5 % concentration. Significant recovery of Cu by tartaric acid might be due to the formation of copper-tartrate complex in the solution and thus enhanced solubilization of the metal ions [10]. Citric acid showed maximum affinity for Mg extraction from brown shale (72.09 %). The values of leached metal by tartaric, malic and oxalic acid were 62, 44 and 67% respectively.

For Mn solubilization, tartaric acid showed significant potential and solubilized 77 % Mn with 0.5 % acid concentration (Fig. 4) while oxalic acid solubilized 52 % Mn with same acid concentration and 72 % of Mn was solubilized with 0.5 % concentration of citric acid. 60 % manganese was solubilized with 0.5 % concentration of malic acid. These results suggested that optimal metal solubilization require controlled hydrogen ion concentration. Increased H^+ concentration resulted in less effective metal dissolution [11].

Production of Organic Acids by Penicillium notatum

Regarding fungal growth characteristics, light yellow colored, tiny beads appeared after about 20 h of incubation in shaking flasks. Size and number of beads increased as the time progressed. The appeared hyphae were branched, scale like with knobbyends. On the condensed mass, small hyphae appeared and the color of hyphae changed from light yellow to bluish green at the end of the growth period. This bluish green color was of conidia because the hyphae of Penicillium are colorless in the inoculated flasks [12]. At the end of growth period, decrease in pH due to organic acid production was observed in all inoculated flasks except control flask contained no substrate. Maximum biomass production (4.78 %) was noted in the medium having glucose as substrate followed by the medium having molasses (3.89 %) and bread crumbs (3.35 %) as clear from Table-1. High yield of biomass (wet) production is complementary to the acids production. Microbial oxidation of substrate is an acid producing system. During the growth studies of *P. notatum*, substrate undergoes microbial oxidation which results in production of citric, oxalic, tartaric and malic acids that play fundamental role in the environmental mobility of metals (Table-1) [13, 14]. Some of these acids act as complexing agent with 2 or more electron donors for metal ions. Citric, oxalic and tartaric acid have chelating properties and have more affinity for Cr, Ni and Zn in the soil [15-17].

Table-1: Concentration of organic acids in fermented media after 15 days of fungal growth (before leaching as well as after leaching)

		Organic acids (mg/L w/v)			
	pH of fermented media	Oxalic acid	Tartaric acid	Citric acid	Malic acid
		Glucose			
Before leaching	2.8	4.352	N.D	15.80	2.170
After leaching	8.5	0.080	N.D	0.028	0.10
		Molasses			
Before leaching	3.2	8.32	70.05	0.662	1.240
After leaching	7.2	N.D	35.828	N.D	N.D
		Bread crumbs			
Before leaching	3.9	0.480	N.D	0.532	N.D
After leaching	5.4	N.D	N.D	N.D	N.D

(Standard error for experimental samples is ≤±0.015, for control is ≤±0.025)

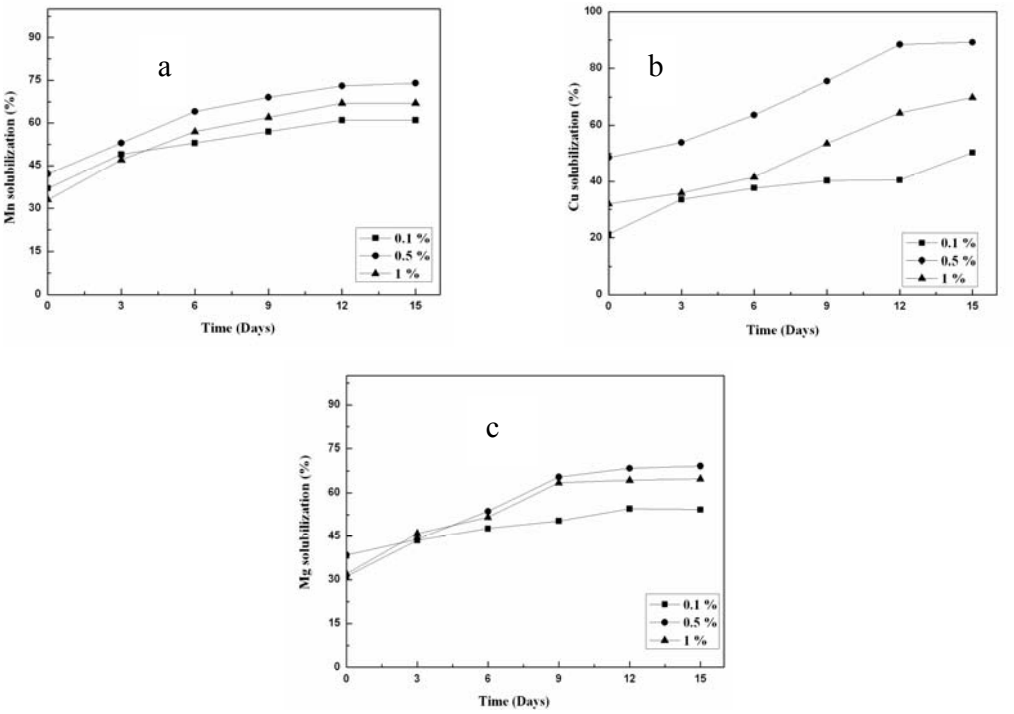

Fig. 1: Citric acid solubilization of metal ions (standard error is ≤±0.08).

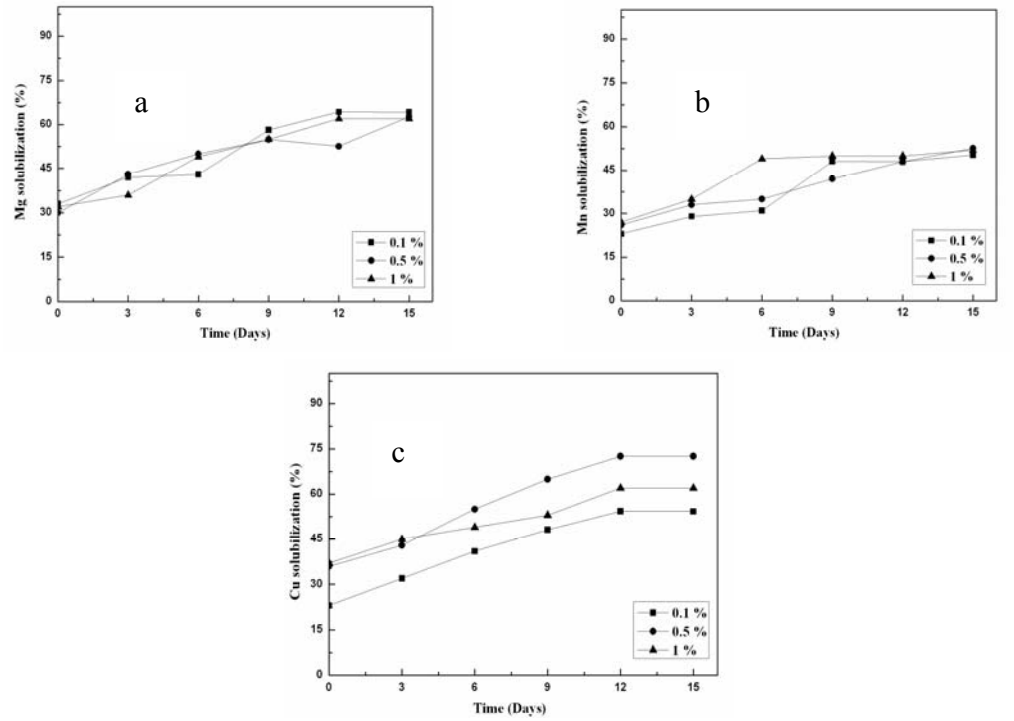

Fig.2: Oxalic acid solubilization of metal ions (standard error is ≤±0.08).

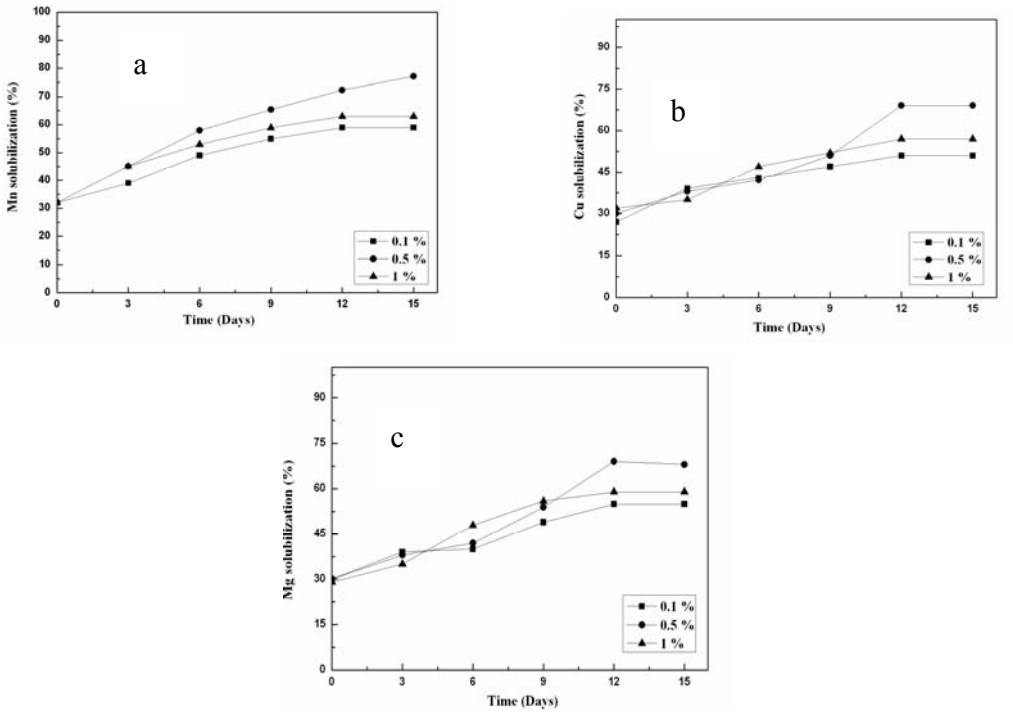

Fig.3**:** Tartaric acid solubilization of metal ions (standard error is ≤±0.08).

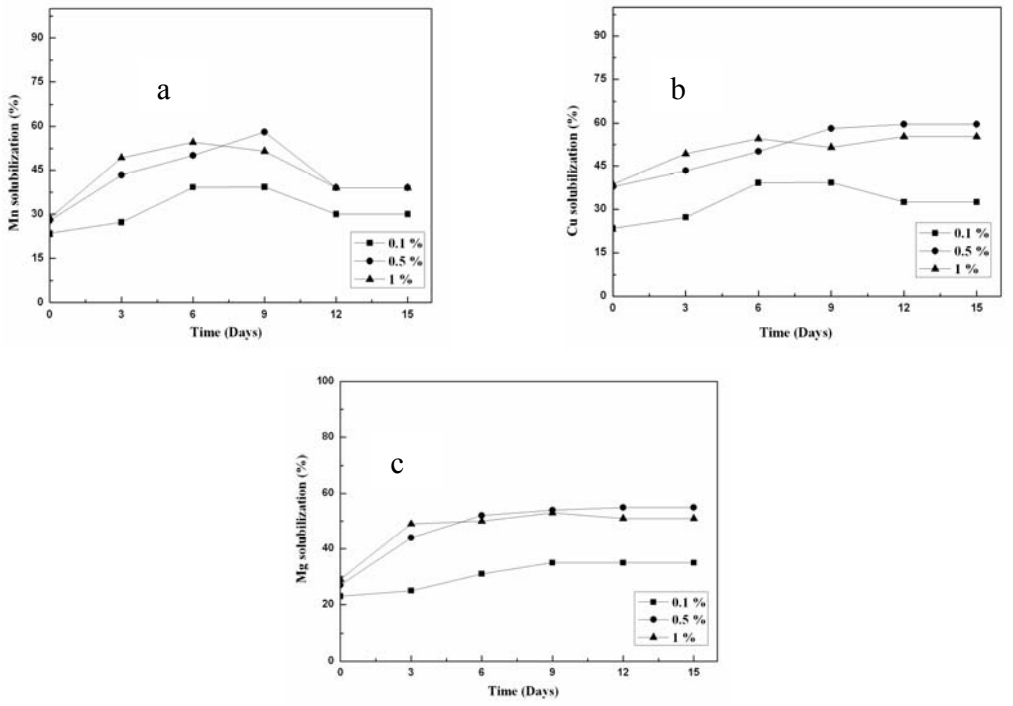

Fig. 4: Malic acid solubilization of metal ions (standard error is ≤±0.08).

To investigate the effectiveness and utilization of organic acids in metal solubilization during leaching process, organic acids produced in all media were collected before leaching (after 15 days of growth period) as well as that of sample collected on 36th day of leaching period and were analyzed by HPLC. The HPLC data of organic acids are shown in Table-1. It is obvious from the HPLC data that major acid produced by *P. notatum* was citric acid which is followed by oxalic acid in all analyzed samples after 15 days of incubation. However, malic acid produced only in medium 1 and 2 whereas tartaric acid found only in medium 2. Some of these acids are good metal chelators which can form complex with metal ions.

pH profiles of leached suspensions in all the three media i.e. glucose, molasses and bread crumbs is shown in Table-1. It can be seen from table that initial pH (4.5) of all media inoculated with *P. notatum* was decreased due to the formation of organic acids by the microbial oxidation of carbon source by metabolic activity [18, 19]. The lowest decrease in pH up to 2.8 was attained in case of glucose followed by molasses (3.2) and bread crumbs (3.9). Similar findings have been reported by Willscher and Bosecke [20]. Similarly maximum biomass production yield was obtained with glucose as substrate followed by molasses and bread crumbs as shown in Table-2.

Table 2: Maximum biomass production

Substrates	Maximum Biomass (%)
Glucose	4.78 %
Molasses	3.89 %
Bread Crumbs	3.35 %

(Standard error is ≤±0.02)

Bioleaching of Metals from Brown Shale

Bioleaching with fungus *P. notatum* was carried out to determine if the acids produced by fungus enhance metal leaching in shaken conditions. Glucose was taken as standard substrate. *P. notatum* was found to be more efficient in solubalizing copper, by using glucose as substrate, compared to magnesium (Mg) and manganese (Mn).This may be due to the high leaching activity of secondary metabolites produced by *P. notatum* in the presence of glucose. Cu solubilized up to 91.55 % with shaking condition, using glucose as substrate up to 24 days of incubation. Maximum recovery of copper in case of molasses was (88.43 %) up to 24 day of incubation which is followed by bread crumbs (58.2 %) as substrates as shown in Fig. 5. While in case of un-inoculated controls the percent recoveries of metals very negligible.

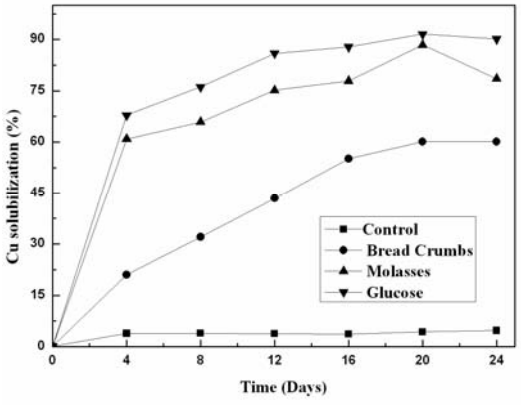

Fig.5: Bioleaching of copper ions in different media (standard error is ≤±0.07)

The highest solubilized magnesium concentration (79.83 %) was achieved with glucose as a substrate. Similar observations are reported earlier [21, 22]. Maximum Mg leach ability exhibited by the *P. notatum* when molasses was used as substrate was about 62.64 % of Mg from brown shale ore in 24 days. A decrease in the bioleaching activity was observed at the late stages of bioleaching of Mg from ore. It might be due to the reason that during bioleaching processes, co-precipitation of metals with mineral phases reduced the leaching efficiencies as reported by Hosseini *et al.* [23]. In addition, the precipitation of compounds present in the leachates on the minerals to be leached can make the solid material inaccessible for fungal leaching. By using bread crumbs as substrate a maximum Mg recovery of was about 47.81 % as shown in Fig. 6. With glucose and molasses as substrate, maximum recovery of Mn^{2+} was 62.59 and 71.46 % as shown in Fig. 7. Significant solubilization of Mg might be due to the acidic environment of the medium and chelating capacity of the organic acids (23). The highest recovery of manganese achieved with bread crumbs was 41.83 %.

Mass Balances

Analysis of residual solids after leaching was carried out to perform a mass balance for Cu, Mn and Mg. The amount of Cu was determined in the residual solids and then added to the maximum

amount of Mn and Mg solubilized. This value was then compared to the initial amount of Cu, Mn and Mg in the raw sample. On the average, some amounts of Cu (23 %), Mn (12 %) and Mg (5.5 %) have not been recovered. During sample preparation and filtration some of the undissolved salts might be lost.

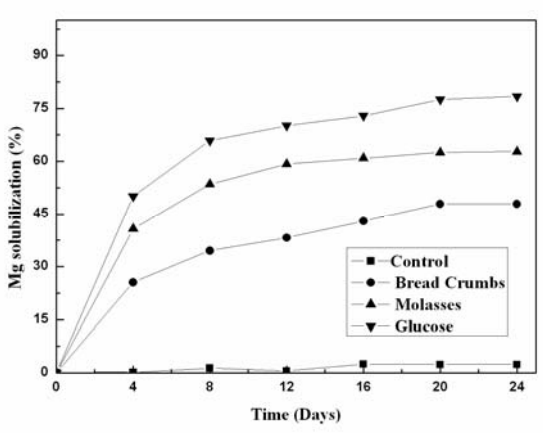

Fig. 6: Bioleaching of magnesium ions in different media (standard error is ≤±0.065)

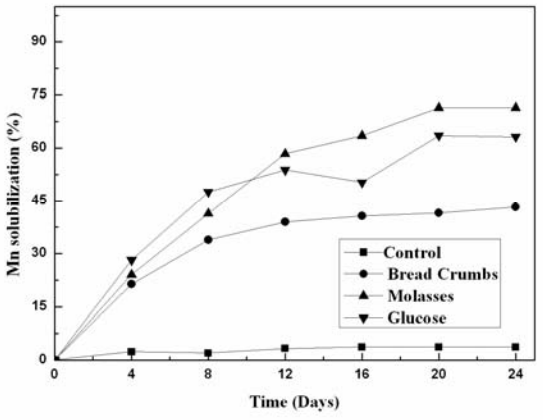

Fig. 7: Bioleaching of manganese ions in different media (standard error is ≤±0.065)

Experimental

Brown Shale Sample

Brown shale ore sample was obtained from Mineralogical Centre Chakwal, Pakisan. It was alkaline (pH 7.5) and insoluble in water. The sample was oven dried ground to 200 mesh size by ASTM sieving machine. Elemental analysis was carried out by atomic absorption spectrophotometry (Perkin Elmer, AAnalyst 300). Raw sample of brown shale mainly composed of (mg/kg) Ca (869), Al (365.4), Mg (247.4), Cu (147), Mn (95.8) and Ni (43.7).

Fungal Strain and Growth Conditions

P. notatum was obtained from culture collection of University of Agriculture, Faisalabad, Pakistan and then growth wan maintained in liquid medium, the culture medium consisted of (g/L): KH_2PO_4, 5.0; NH_4NO_3, 2.0; $(NH_4)_2SO_4$, 4.0; $MgSO_4.7H_2O$, 0.2; peptone, 2.0; trisodium citrate, 2.5; yeast extract, 1.0 and made volume up to 1000 mL with distilled water [24]. Nine sets of 250 mL flasks were prepared containing 100 mL of liquid medium each in triplicate samples. Medium in each flask was autoclaved at 121°C for 15 min. After sterilization, 5 % of given substrate was added in each flask then inoculated with 1 mL of *P. notatum* spore suspension as inoculum (approximately 1.8 $\times 10^6$ spores mL^{-1}). All the flasks were sealed with removable cotton and incubated in an orbital shaker (Gellen Kamp, England) at 28 °C at an operating speed of 120 rpm for 15 days. Solid media for growth consisted of PDA (3.9 %). It was incubated (Incubator, Sanyo, Germany) for 72 h at 28 °C to produce an adequate number of spores. The spores were counted using Petroff-Hausser counting chamber.

Sources of Substrates

Different substrates i.e. glucose, molasses and bread crumbs (5 %) were used under shaking conditions. Glucose was taken as standard during this study. Glucose and molasses were sterilized at 120 º C for 15 min prior to bioleaching experiments while bread crumbs were dried, grind and sterilized.

Chemical Leaching

To investigate metal ions solubilization in correlation with various concentrations of oxalic, tartaric and citric acids (0.1, 0.5 and 1.0 %), chemical leaching tests were performed. Triplicate samples with different concentration of organic acids (3 % pulp density) were subjected to shaking for a period of 15 days. Liquid samples were filtered and analyzed for metal ions by atomic absorption spectrophotometry (AAS). pH of the liquid samples was monitored after every 48 h of leaching period.

Leaching of Metal ions from Organic Acids produced by Penicillium notatum

After incubation period of 15 days, supernatant liquor of microbial culture from shaking conditions, containing glucose, molasses and bread crumbs (2 %), were collected, centrifuged (8000 rpm for 10 min at 15 °C) and filtered to remove solids biomass before HPLC analysis for the determination of organic acid metabolites. Supernatants containing organic acid metabolites were used to leach the metal ions from shale residue. Then 3 % (w/v) of shale ore sample was added to leaching media containing flasks and incubated in an orbital shaker to keep everything in homogeneous slurry form at 28 °C and 120 rpm for 24 days. Samples were collected after every 2nd day of leaching period, pH was monitored after every 24 hours and supernatant were analyzed for metal ions analysis.

Analysis of Organic Acids

Analyses of metabolites (e.g. citric, oxalic, tartaric and malic acids) of all media were performed by following the modified HPLC method as described by Escobal *et al.* [25]. After centrifugation and filtration, samples were vortexed before HPLC analysis. The mobile phase consisted of 0.25 % of acetic acid, was filtered and sonicated to remove if any of the suspended particles. An HPLC (Sykam GmbH, Kleinostheim, Germany) equipped with S-1121 dual piston solvent delivery system and S-3210 UV/VIS diode array detector and software package for data acquisition was used. A 20 µL of filtered sample was injected in to an analytical Hypersil (Thermo Hypersil, GmbH, Germany) ODS reverse phase (C) column (250 4.6 mm; 5 µm particle size) fitted with a C guard column. The chromatographic separation was performed by isocratic elution of the mobile phase at a flow rate of 1.0 mL/min at 30 °C. Detection was performed at wavelength of 254 nm. Organic acids were identified by comparing the retention times and quantified on the basis of peak area. The peak areas were recorded and calculated by a computer with, chromatography data acquisition and integration software (SRI Instrument, Torrance, California, USA).

Metal ions Analysis

Samples collected on every fifth day of leaching period, were subjected to metal ions analysis by atomic absorption spectrophotometry (Perkin Elmer, Analyst 300). At the end of leaching period of each medium, residue samples were washed with water three times and then oven dried. Residues were subjected to wet digestion process by using nitric acid and hydrogen peroxide.

Statistical Analysis

All experiments of the samples before and after leaching were performed in triplicate and the results were reported with standard errors [26].

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